

# Key Differences in Molecular Complexes of the Cholecystokinin Receptor with Structurally Related Peptide Agonist, Partial Agonist, and Antagonist

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## ABSTRACT

The molecular basis of docking of receptor ligands having differences in biological activity and their subsequent effects on receptor conformation represent areas of great interest. In this work, we focus on the sulfated tyrosyl residue in position 27 of cholecystokinin (CCK) and its spatial approximation with the type A CCK receptor residue Arg<sup>197</sup> that has been predicted from mutagenesis experiments. We have examined the requirement for sulfation of this residue in a series of structurally related peptide agonists, partial agonists, and antagonists using assays of receptor binding and biological activity. Whereas sulfation of CCK position 27 was critical for affinity and potency

of a full agonist, it had progressively less effect as the biological activity of the ligand was reduced. It had an intermediate effect on the partial agonist and no effect on the antagonist. In addition, photoaffinity labeling was used to determine the spatial approximations between the receptor and residue 27 of the agonist and antagonist in this series. Direct photoaffinity labeling with a full agonist probe confirmed the spatial approximation of ligand residue 27 and receptor residue Arg<sup>197</sup> in the active complex. Of note, the analogous antagonist probe labeled a distinct region within the receptor amino terminus, confirming a key structural difference in active and inactive complexes.

As the largest group of plasma membrane receptors, G protein-coupled receptors (GPCRs) offer targets for a wide variety of drugs. Understanding the molecular basis of ligand binding and agonist-induced receptor activation would greatly facilitate the design of new therapeutic agents that target these receptors. However, only one comprehensive, high-resolution, three-dimensional structure of a member of this superfamily, that of rhodopsin, has been published (Palczewski et al., 2000). By analogy, rhodopsin's structure can provide general information on the structure of other GPCRs in the class A family. However, there are myriad natural ligands for GPCRs, which include photons, biogenic amines, peptides, and glycoproteins, indicating that although all GPCRs seem to share the seven-transmembrane helical bundle, the receptor-ligand interactions may differ substantially depending on the ligand and receptor being investigated. Without the availability of high-resolution, three-

dimensional structure information, we have become dependent on less direct techniques such as receptor mutagenesis and photoaffinity labeling.

Using these techniques, we have examined the molecular basis of ligand-induced activation of the type A cholecystokinin (CCK) receptor by the peptide hormone CCK (Ji et al., 1997; Hadac et al., 1998, 1999; Dong et al., 1999; Ding et al., 2001, 2002; Harikumar et al., 2002). Through this receptor, CCK stimulates gallbladder contraction, pancreatic exocrine secretion, and gut motility and induces postcibal satiety. There are variable lengths of CCK, from 8 to 72 amino acids, all sharing a common carboxyl-terminal domain. The carboxyl-terminal heptapeptide is the minimal sequence that elicits a response with full efficacy and potency at the CCK receptor (Jensen et al., 1982). The full agonist, CCK, can be changed into a partial agonist by changing the carboxyl-terminal phenylalanine-amide in position 33 into a phenylethyl ester (OPE). Further modification of the peptide, by changing the L-tryptophan residue in position 30 to a D-tryptophan residue, results in a peptide antagonist (D-Trp-OPE). Incorporation of photolabile residues within the pharmacophore of CCK has allowed for the determination of spatial approximations between residues of CCK and the recep-

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**ABBREVIATIONS:** GPCR, G protein-coupled receptor; CCK, cholecystokinin; *p*NO<sub>2</sub>-Phe, *para*-nitro-phenylalanine; (*p*NO<sub>2</sub>-Phe)<sup>27</sup> agonist probe, D-Tyr-Gly-[(*p*NO<sub>2</sub>-Phe)<sup>27</sup>,Nle<sup>28,31</sup>]CCK-26–33; (*p*NO<sub>2</sub>-Phe)<sup>27</sup> antagonist probe, D-Tyr-Gly-[(*p*NO<sub>2</sub>-Phe)<sup>27</sup>,Nle<sup>28,31</sup>,D-Trp<sup>30</sup>]CCK-26–32-phenylethyl ester; CHO, Chinese hamster ovary; CHO-CCKR, Chinese hamster ovary cell lines stably expressing the wild-type rat type A cholecystokinin receptor; STI, soybean trypsin inhibitor; KRH, Krebs-Ringer/HEPES; CNBr, cyanogen bromide; Endo F, endoglycosidase F.

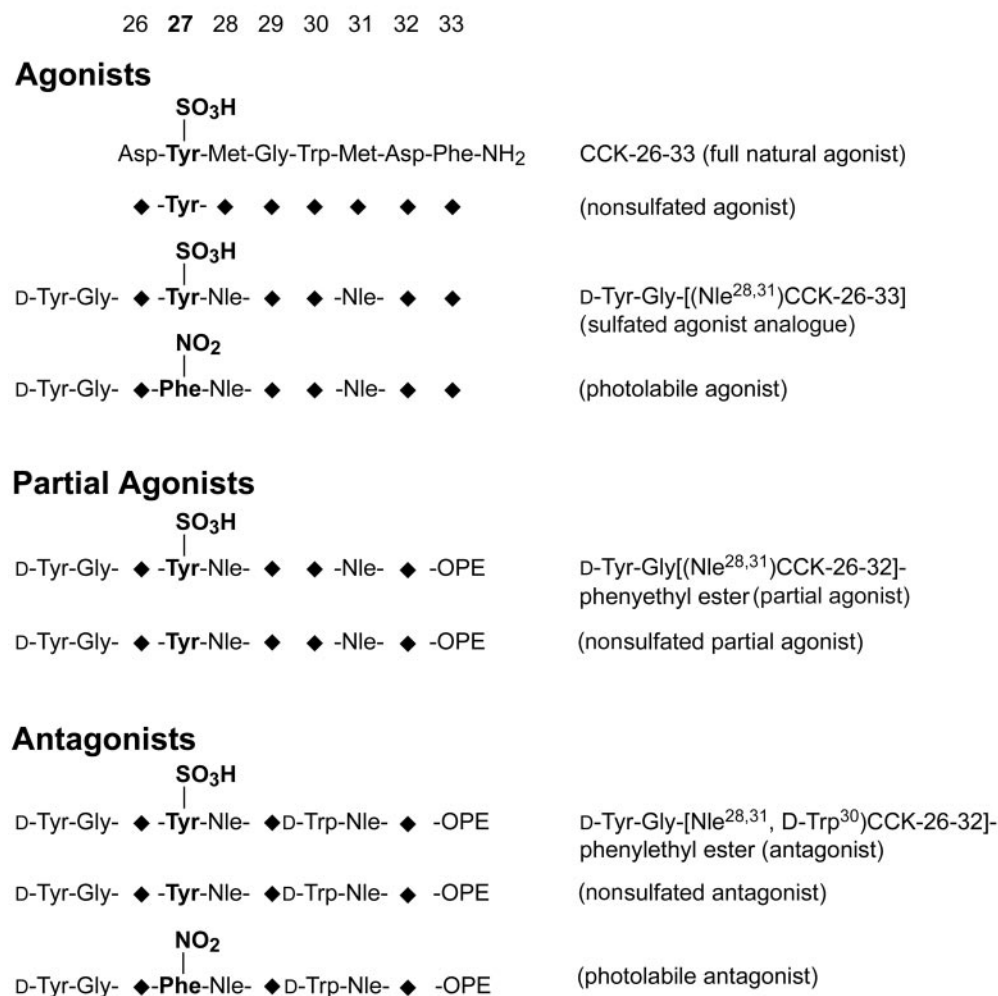
tor. These spatial constraints have been used to develop a molecular model of the complex of CCK bound to its receptor (Ding et al., 2002).

The post-translational addition of sulfate to the tyrosine residue in position 27 of CCK is critical for high-affinity binding and biological activity of CCK at its receptor. Desulfation of natural CCK results in a loss in affinity and potency of 3 to 4 orders of magnitude (Innis and Snyder, 1980; Jensen et al., 1982). It has been predicted that this acidic sulfated tyrosine residue in CCK interacts with basic Arg<sup>197</sup> in the second extracellular loop of the CCK receptor, as determined from receptor mutagenesis data (Gigoux et al., 1999) and from a combination of complementary changes in receptor and ligand (Ding et al., 2002). However, this has not been directly demonstrated or confirmed previously. In addition, the sulfation requirement for structurally related peptide partial agonists and antagonists and the spatial relationship between position 27 of these ligands and the receptor have not been studied. In the current work, we used structure-activity relationships to examine the requirement for tyrosine sulfation in position 27 in a series of structurally related agonists, partial agonists, and antagonists. Furthermore, we used intrinsic photoaffinity labeling to directly determine spatial approximations between the CCK receptor and residue 27 of the probes in this series and demonstrated structural differences in the CCK receptor-ligand complexes when docked with agonist versus antagonist.

## Materials and Methods

**Peptide Synthesis.** A series of CCK analogs were synthesized by solid- and solution-phase techniques, as described previously (Powers et al., 1988a,b) (Fig. 1). Modifications of these peptides included replacement of the Tyr-sulfate residue in position 27 with nonsulfated-Tyr and photolabile *para*-nitro-phenylalanine (*p*NO<sub>2</sub>-Phe). In partial agonist and antagonist peptides, Phe<sup>33</sup> was replaced with a phenylethyl ester. Antagonist peptides were further modified by replacing L-Trp<sup>30</sup> with a D-Trp residue. Each peptide also had an amino-terminal extension of Tyr-Gly to provide a site for radioiodination, and Met residues were replaced with oxidation-resistant norleucine (Nle) residues. The radioligand D-Tyr-Gly-[(Nle<sup>28,31</sup>)CCK-26-33] that was used for receptor binding assays, the photolabile agonist D-Tyr-Gly-[(*p*NO<sub>2</sub>-Phe)<sup>27</sup>,Nle<sup>28,31</sup>)CCK-26-33] [(*p*NO<sub>2</sub>-Phe)<sup>27</sup> agonist probe], and photolabile antagonist, D-Tyr-Gly-[(*p*NO<sub>2</sub>-Phe)<sup>27</sup>,Nle<sup>28,31</sup>,D-Trp<sup>30</sup>)CCK-26-32]-phenylethyl ester [(*p*NO<sub>2</sub>-Phe)<sup>27</sup> antagonist probe], were radioiodinated oxidatively with Na<sup>125</sup>I upon exposure to the solid-phase oxidant *N*-chloro-benzenesulfonamide (ODO beads; Pierce, Rockford, IL), for 15 s and purified by reverse-phase high-performance liquid chromatography to yield specific radioactivity of 2000 Ci/mmol (Powers et al., 1988a).

**Receptor Preparations.** Chinese hamster ovary (CHO) cell lines stably expressing the wild-type rat type A CCK receptor (CHO-CCKR) (Hadac et al., 1996), and M195L (Gigoux et al., 1998), A204C (Ding et al., 2003) and V342M (Hadac et al., 1998) CCK receptor mutants that have been established and characterized previously were used as sources of receptors for the current study. An additional



**Fig. 1.** Primary structures of CCK analogs used in this study.

CHO cell line that expressed a CCK receptor mutant construct in which Leu<sup>104</sup> was mutated to Met (L104M) was established for this work with the use of methods that we described previously (Hadac et al., 1996). This construct was prepared using an oligonucleotide-directed approach with the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA), with the products verified by direct DNA sequencing.

Cell lines were grown in monolayers in flasks containing Ham's F-12 medium with 5% Fetal Clone-2 (Hyclone Laboratories, Logan, UT) in a 5% CO<sub>2</sub> humidified environment at 37°C. Cells were passaged approximately twice weekly. Enriched plasma membranes were prepared as described previously (Hadac et al., 1996). In brief, after mechanical harvesting, cells were suspended and sonicated in 0.3 M sucrose containing 0.01% soybean trypsin inhibitor (STI) and 1 mM phenylmethylsulfonyl fluoride. The sucrose concentration of the cell suspension was increased to 1.3 M, and this suspension was overlaid with 0.3 M sucrose before centrifugation at 225,000g for 1 h at 4°C. The membrane layer was then removed and diluted with water. Centrifugation of this mixture at 225,000g for 30 min at 4°C resulted in a pellet that was resuspended in Krebs-Ringer/HEPES (KRH) solution containing 25 mM HEPES, pH 7.4, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 104 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, and 1.2 mM MgSO<sub>4</sub> with 1 mM phenylmethylsulfonyl fluoride and 0.01% STI and stored at -80°C.

**Receptor Binding and Biological Activity.** As described previously (Hadac et al., 1996), binding of ligand to the CCK receptor was examined by incubation of 2 to 5 µg of membrane protein with a constant amount (5 pM) of radioligand, [<sup>125</sup>I]-D-Tyr-Gly-[(Nle)<sup>28,31</sup>]-CCK-26-33], that has been fully characterized and validated previously (Pearson et al., 1987a). This was done in the absence and presence of increasing concentrations (from 0 to 1 µM) of the unlabeled ligand of interest. The reaction was performed for 1 h at room temperature in KRH medium containing 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin (final volume, 0.5 ml). A Skatron cell harvester (Molecular Devices, Sunnyvale, CA) and receptor-binding glass-fiber filtermats were used to separate free probe from receptor-bound probe. Quantification of bound radioligand was done with a γ-spectrometer. Nonspecific binding was determined in the presence of 1 µM CCK and represented less than 20% of total bound counts per minute. Binding curves were analyzed and plotted using the nonlinear regression analysis routine for radioligand binding in the Prism software package (GraphPad Software, San Diego, CA).

The biological activity of the ligands was characterized by examining the ability of ligands to stimulate intracellular calcium release. CCK receptor-bearing CHO-CCKR cells were lifted with nonenzymatic cell dissociation solution (Sigma Chemical, St. Louis, MO) and loaded with Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR). Cells were then washed and resuspended in KRH with 0.01% STI and 0.2% bovine serum albumin. For each peptide concentration tested, approximately 2 million cells were treated with peptide, and fluorescence was measured by a PerkinElmer LS50B luminescence spectrometer (PerkinElmer Life and Analytical Sciences, Boston, MA). Calcium concentrations were calculated as described previously (Gryniewicz et al., 1985) after excitation at 340 and 380 nm with emissions measurement at 520 nm. Treatment of cells with antagonist alone elicited no calcium response. Inhibition of the CCK-induced calcium response by antagonists was demonstrated by treating cells in the presence of 10<sup>-10</sup> M CCK with varied concentrations of antagonist. Peak intracellular calcium concentrations were used to determine the concentration-dependence of responses.

**Photoaffinity Labeling of CCK Receptor.** Covalent labeling of the CCK receptor was achieved as described previously (Ding et al., 2001). In brief, 50 µg of enriched receptor-bearing membranes from CHO-CCKR cells were incubated with 0.1 nM [<sup>125</sup>I]-(*p*NO<sub>2</sub>-Phe)<sup>27</sup> agonist or antagonist in the presence of increasing concentrations of CCK (from 0 to 1 µM) in KRH buffer (final volume, 0.5 ml) in the dark for 1 h at room temperature. This was then exposed to photolysis for 30 min at 4°C in a Rayonet photochemical reactor (Southern New England Ultraviolet Company, Hamden, CT) equipped with

3000-Å lamps (approximately 90% irradiance delivered between wavelengths of 290 and 330 nm). Membrane proteins (50 µg) were then either directly applied to a 10% SDS-polyacrylamide gel for electrophoresis or solubilized with 1% Nonidet P-40 in KRH medium before wheat-germ agglutinin-agarose affinity chromatography and subsequent SDS-polyacrylamide gel electrophoresis. The labeled receptor bands were visualized by autoradiography. To prepare labeled receptor in larger scale for further peptide mapping, a larger amount of receptor-bearing membranes (200 µg) and [<sup>125</sup>I]-(*p*NO<sub>2</sub>-Phe)<sup>27</sup> agonist or antagonist (0.5 nM) were incubated in the absence of competing CCK before photolysis.

To determine the region of covalent labeling of the receptor by each probe, 200 µg of receptor-bearing membranes were used in batches for photoaffinity labeling. Radiolabeled receptor bands were excised from polyacrylamide gels, eluted with water, lyophilized, ethanol-precipitated, and cleaved with 2.5 mg of cyanogen bromide (CNBr; Pierce) in 70% formic acid, as described in detail previously (Hadac et al., 1998). After cleavage, samples were washed in water and dried in a vacuum centrifuge. The products of cleavage were resolved on a 10% NuPAGE gel (Invitrogen, Carlsbad, CA) and visualized by autoradiography (Ji et al., 1997). Subsequent endoproteinase Lys-C digestion was performed, as we reported previously (Dong et al., 1999). The glycosylation status of the labeled fragment was determined by overnight treatment of the receptor or its fragment with 2 units of endoglycosidase F (Endo F) (Pearson et al., 1987b) in buffer containing 0.1 M sodium phosphate, pH 6.1, 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 1% 2-mercaptoethanol. These products were separated by electrophoresis on either an SDS-polyacrylamide gel (for intact receptor) or a NuPAGE gel (for receptor fragments), and labeled bands were visualized by autoradiography.

#### Identification of the Covalently Labeled Receptor Residue.

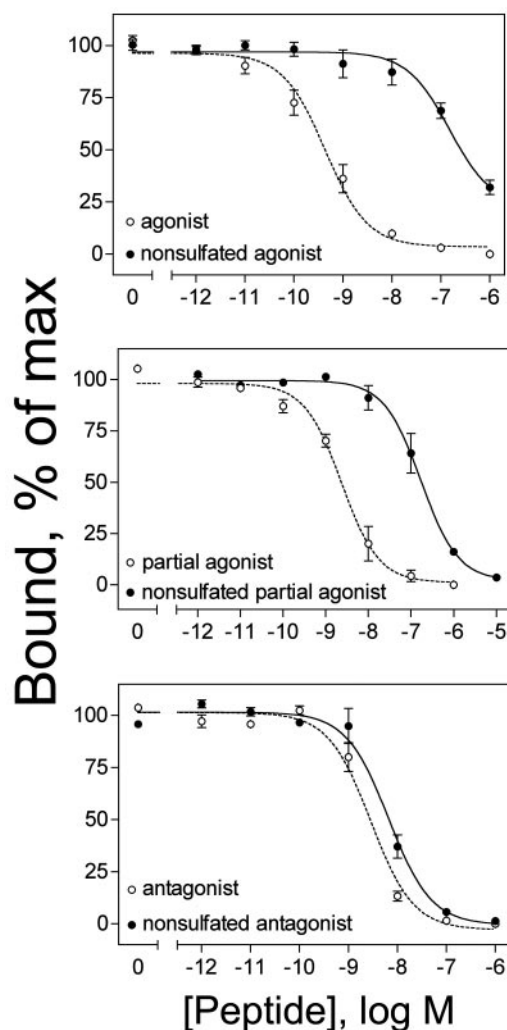
Once it was clear which receptor fragment was covalently labeled, radiochemical sequencing was used to identify the precise residue of attachment of the photolabile probe. For labeling with the (*p*NO<sub>2</sub>-Phe)<sup>27</sup> full-agonist probe, the A204C CCK receptor mutant (Ding et al., 2003) was used in the radiochemical sequencing experiments. Before CNBr cleavage, the amino termini of the labeled receptor and the attached probe were blocked by acetylation with acetic anhydride using methods that we described previously (Dong et al., 2004). CNBr cleavage was then performed to expose a free amino group within the released receptor fragment that would be amenable for sequencing. The radiolabeled fragment from CNBr cleavage of the A204C CCK receptor mutant was purified and coupled to *N*-(2-aminoethyl-1)-3-aminopropyl glass beads (Sigma) through the thiol group of Cys<sup>204</sup>. Manual cycles of Edman degradation were repeated, as has been reported previously (Ji et al., 1997), and the radioactivity released in each cycle was quantified in a γ-spectrometer.

## Results

**Effect of Tyrosine Sulfation on Ligand Binding.** The requirement of Tyr<sup>27</sup> sulfation was examined by looking at the binding of sulfated and nonsulfated structurally related CCK receptor agonists, partial agonists, and antagonists to the CCK receptor (structures shown in Fig. 1). Sulfated agonist had a binding affinity 3 orders of magnitude greater than that of nonsulfated agonist (Fig. 2, top). The nonsulfated partial agonist had a smaller loss in affinity of 2 orders of magnitude compared with the sulfated partial agonist (Fig. 2, middle). There was no significant difference in affinities of the sulfated and nonsulfated antagonists (Fig. 2, bottom). Thus, ligand sulfation is critical for agonist binding to the CCK receptor, but, as the ligand is changed from agonist to partial agonist to antagonist, the requirement for sulfation of Tyr<sup>27</sup> is decreased and eliminated, respectively.



**Effect of Sulfation on Biological Activity.** Because of the difference in the need for ligand sulfation for binding of agonists and antagonists, we investigated whether there was a similar difference in biological activity. The ability of the sulfated and nonsulfated agonists to stimulate calcium release was examined. We found that the nonsulfated full agonist CCK had potency 4 orders of magnitude lower than its sulfated analog (Fig. 3, top). The nonsulfated agonist remained a full agonist, as demonstrated by the similar calcium response, to its maximum, relative to that stimulated by sulfated CCK. The ability of sulfated and nonsulfated antagonists to inhibit a CCK-stimulated calcium response was also examined. After ensuring that these compounds alone did not elicit a calcium response, cells were pretreated

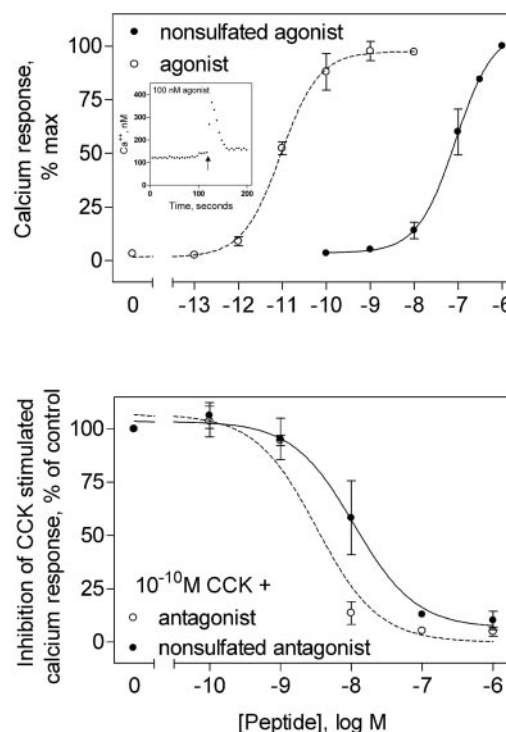


**Fig. 2.** Competition for CCK receptor binding. Shown are competition-binding curves of peptide agonists (top), partial agonists (middle), and antagonists (bottom) that are sulfated or nonsulfated at Tyr<sup>27</sup>. Desulfation of agonist resulted in a loss in affinity for the full agonist of 3 orders of magnitude, whereas desulfation of partial agonist resulted in a loss in affinity of 3 orders of magnitude. There was no significant sulfation-dependent change in antagonist affinity observed. Values are expressed as means  $\pm$  S.E.M. of data from a minimum of three independent experiments. The absolute values for maximal binding in these experiments were  $2696 \pm 156$  cpm for agonist,  $2700 \pm 222$  cpm for nonsulfated agonist,  $2689 \pm 157$  cpm for partial agonist,  $2710 \pm 150$  cpm for nonsulfated partial agonist,  $2737 \pm 162$  cpm for antagonist, and  $2766 \pm 120$  cpm for nonsulfated antagonist.

with candidates for antagonist activity 30 s before the addition of  $10^{-10}$  M CCK. Sulfation did not have a significant effect on the ability of the antagonists to inhibit the calcium response (Fig. 3, bottom). Thus, similar to binding, sulfation of Tyr<sup>27</sup> drastically affected agonist potency but did not affect the ability of an antagonist to inhibit a biological response.

**Characterization of Photolabile CCK Analogs.** To directly explore the spatial approximation of the position 27 residue in agonist and antagonist analogs with the CCK receptor, we developed photolabile probes in which Tyr<sup>27</sup> was replaced with a photolabile *p*NO<sub>2</sub>-Phe residue (Fig. 1). We postulated that the differences in sulfation requirement for binding and biological activity of agonist and antagonist would reflect differences in the interactions between this residue within the ligands and the CCK receptor. We hoped to show these differences with our (*p*NO<sub>2</sub>-Phe)<sup>27</sup> photolabile probes. Figure 4 shows the binding characteristics and biological activities of these probes. Both the agonist and antagonist photolabile probes competed for and displaced the binding of the radioligand to the CCK receptor in a concentration-dependent manner (Fig. 4, top). Furthermore, the photolabile agonist elicited a calcium response in a concentration-dependent manner (Fig. 4, middle), and the photolabile antagonist, having no endogenous agonist activity, inhibited the CCK-stimulated calcium response in a concentration-dependent manner (Fig. 4, bottom).

**Photoaffinity Labeling of the CCK Receptor.** After we characterized the photolabile probes, we used them to covalently label the CCK receptor. Both the photolabile agonist



**Fig. 3.** Intracellular calcium responses induced by CCK analogs in CHO-CCKR cells. Desulfation of agonist resulted in a loss in potency of 4 orders of magnitude to stimulate an intracellular calcium response (top). In contrast, nonsulfated antagonist did not demonstrate a significant shift in its ability to inhibit a CCK-stimulated biological response compared with sulfated antagonist (bottom). Values are expressed as means  $\pm$  S.E.M. of data from a minimum of three independent experiments. Basal level of intracellular calcium was  $142 \pm 16$  nM, and maximal levels of stimulation reached  $383 \pm 32$  nM for agonist and  $376 \pm 29$  nM for nonsulfated agonist.

and the photolabile antagonist specifically labeled the CCK receptor, with the radiolabeled receptor bands migrating on SDS-polyacrylamide gels at approximately  $M_r = 85,000$  to  $95,000$  (Figs. 5 and 6). Labeling of these bands was inhibited in a concentration-dependent manner with CCK (Figs. 5 and 6). Deglycosylation of the receptor with Endo F shifted the migration of the band to approximately  $M_r = 42,000$ , which is the position of the core protein. Membrane from the parental cells not bearing CCK receptors did not get labeled (Fig. 5 and data not shown).

**Identification of the Site of CCK Receptor Labeling with the Agonist Probe.** CNBr, which cleaves at the carboxyl side of Met residues within a protein sequence, was used to provide a first indication of the domain of labeling by the  $(pNO_2\text{-Phe})^{27}$  agonist probe. The CCK receptor contains 15 Met residues, and theoretically, CNBr cleavage of the

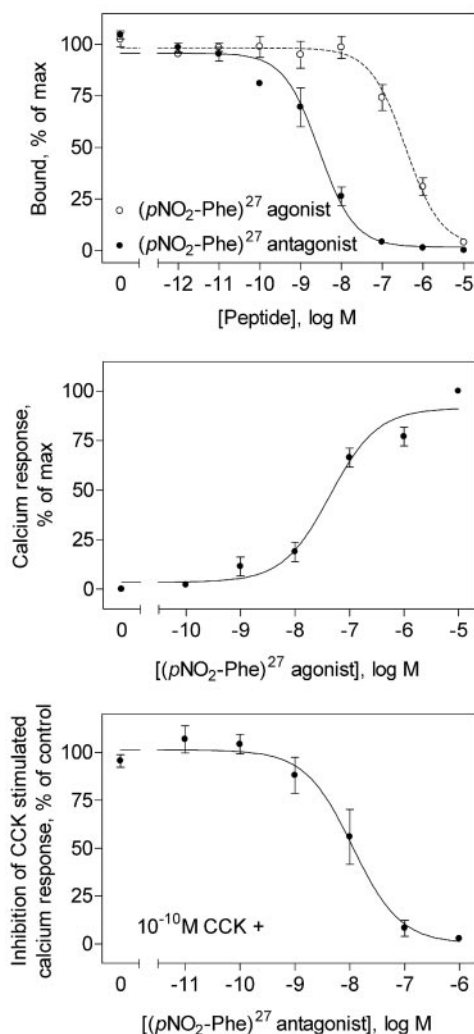
CCK receptor yields 16 fragments ranging in molecular mass from 0.1 to 9.9 kDa, with two fragments containing potential sites of N-linked glycosylation (Fig. 7).

As shown in Fig. 7, CNBr cleavage of the labeled CCK receptor resulted in a radioactive band migrating at approximately  $M_r = 2500$  to  $3500$  on a 10% NuPAGE gel. Treatment with Endo F did not modify the electrophoretic migration of this labeled fragment, suggesting the absence of glycosylation. This ruled out the labeling of the amino terminal fragment (Asn<sup>10</sup>-Met<sup>72</sup>) of the receptor, known to be glycosylated (Ji et al., 1997; Ding et al., 2001). There is another potential site of glycosylation within the second extracellular loop, but it is unclear whether this site is glycosylated when the CCK receptor is expressed in CHO cells. From the electrophoretic migration of the standard proteins and the mass of the attached probe (1277 Da), the mass of the candidate fragment would be expected to be in the range of approximately 1 to 3 kDa. However, small peptides often do not migrate true to their masses, and different standards can migrate differently in this range. Therefore, no fragment from any of the three extracellular loops could be excluded at this stage as a candidate for the domain of labeling.

To further identify which fragment contained the domain of agonist labeling, three receptor mutants were used in which either an additional Met residue was introduced (L104M or V342M) or a naturally occurring Met residue was eliminated (M195L) in each of the three extracellular loop regions. Each of these mutants was stably expressed in CHO cells. Whereas the L104M (data not shown) and V342M (Haddad et al., 1998) mutants were characterized to have binding and biological activity similar to those of the wild-type CCK receptor, the M195L mutant had an affinity approximately 1 order of magnitude lower than that of the wild-type receptor (Gigoux et al., 1998). Each of these constructs was specifically labeled by the  $(pNO_2\text{-Phe})^{27}$  agonist probe (data not shown). The labeled mutant receptors were cleaved by CNBr. As shown in Fig. 8, the CNBr fragments resulting from cleavage of the labeled L104M and V342M receptor mutants migrated on a 10% NuPAGE gel similarly to that from the labeled wild-type receptor. However, the CNBr fragment from cleavage of the labeled M195L receptor mutant resulted in a radioactive band migrating at approximately  $M_r = 5500$ , clearly distinct from that of the CNBr fragment from the wild-type receptor. These data indicate that the fragment spanning the third extracellular loop (Thr<sup>174</sup>-Met<sup>205</sup>) contained the site of labeling for the  $(pNO_2\text{-Phe})^{27}$  agonist probe.

Determination of whether the amino-terminal half (Thr<sup>174</sup>-Met<sup>195</sup>) or the carboxyl-terminal half (Cys<sup>196</sup>-Met<sup>205</sup>) of the second extracellular loop contained the site of agonist labeling was achieved by exposing the labeled fragment from CNBr cleavage of the wild-type receptor to cleavage with endoproteinase Lys-C. As shown in Fig. 8, treatment with this protease did not change the migration of the labeled CNBr fragment, suggesting that the carboxyl-terminal segment of the second extracellular loop (Cys<sup>196</sup>-Met<sup>205</sup>) represented the domain of labeling by this probe.

The definitive identification of the specific site of labeling of the CCK receptor with the  $(pNO_2\text{-Phe})^{27}$  agonist probe was achieved by radiochemical sequencing. For this, the A204C CCK receptor mutant, in which Ala<sup>204</sup> was mutated to Cys (Ding et al., 2003), was used to couple its CNBr fragment Cys<sup>196</sup>-Met<sup>205</sup> through Cys<sup>204</sup> to N-(2-aminoethyl)-1-3-aminopropyl glass beads. This mutant receptor has been charac-



**Fig. 4.** Characterization of photolabile CCK analogs. Shown are competition-binding (top) and biological activity (middle and bottom) curves for  $(pNO_2\text{-Phe})^{27}$ -CCK analogs. Each of these probes displaced the binding of the radioligand to the CCK receptor in a concentration-dependent manner. The absolute values for maximal binding were  $2007 \pm 153$  cpm for the  $(pNO_2\text{-Phe})^{27}$  agonist and  $2036 \pm 203$  cpm for the  $(pNO_2\text{-Phe})^{27}$  antagonist. The  $(pNO_2\text{-Phe})^{27}$  agonist elicited a calcium response in a concentration-dependent manner, whereas the  $(pNO_2\text{-Phe})^{27}$  antagonist had no endogenous agonist activity and inhibited the cellular response to  $10^{-10}$  M CCK in a concentration-dependent manner. Values are expressed as means  $\pm$  S.E.M. of data from a minimum of three independent experiments.

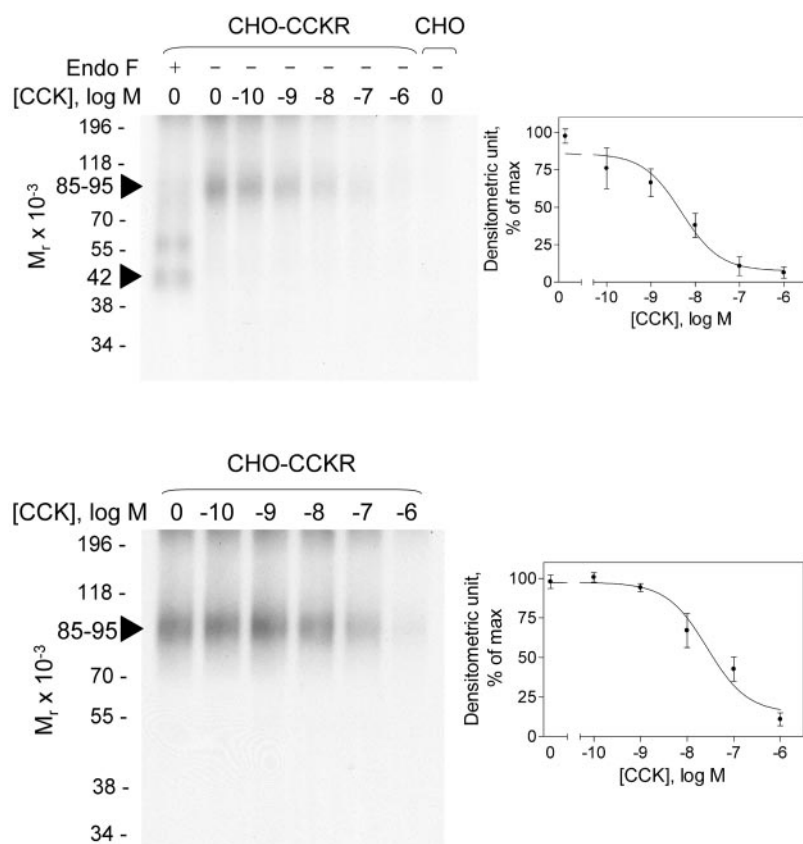
terized to have normal affinity to bind CCK and calcium response to CCK stimulation similar to that of the wild-type receptor (Ding et al., 2003). It was also saturably and specifically labeled by the  $(pNO_2\text{-Phe})^{27}$  agonist probe, and after CNBr cleavage, the resultant fragment migrated similarly to that from the wild-type receptor (data not shown). As shown in Fig. 9, radiochemical sequencing of the labeled fragment Cys<sup>196</sup>-Met<sup>205</sup> identified Arg<sup>197</sup> as the site of labeling by the  $(pNO_2\text{-Phe})^{27}$  agonist probe.

**Identification of the Region of CCK Receptor Labeling with the Antagonist Probe.** In contrast to the data from the agonist probe, the cleavage pattern from the antagonist-labeled CCK receptor was quite different. Digestion of antagonist-labeled receptor with CNBr resulted in a fragment that migrated at approximately  $M_r = 25,000$  (Fig. 10). Unlike the agonist-labeled receptor, treatment of antagonist-labeled receptor with Endo F after CNBr cleavage shifted the labeled band to migrate at approximately  $M_r = 8500$ . This shift was consistent with labeling of the receptor fragment that contains the amino terminus and the first transmembrane domain (Asp<sup>10</sup>-Met<sup>72</sup>). Further localization was accomplished with subsequent endoprotease Lys-C treatment. As shown in Fig. 10, cleavage of the labeled CNBr fragment with this protease yielded a band migrating at approximately  $M_r = 22,000$  that further shifted to approximately  $M_r = 4500$  after deglycosylation with Endo F. Thus, unlike the photolabile agonist that labeled the receptor at Arg<sup>197</sup> within the second extracellular loop, the photolabile antagonist labeled the receptor at a residue between Asn<sup>10</sup> and Lys<sup>37</sup> within the extracellular amino terminus.

## Discussion

In the present study, we examined the requirement for Tyr<sup>27</sup> sulfation in structurally related peptide agonists, partial agonists, and antagonists. We then determined spatial approximations between the type A CCK receptor and residue 27 of related agonist and antagonist ligands. The results suggest that although sulfation in the 27 position of the CCK pharmacophore is necessary for agonist affinity and potency, sulfation has no significant effect on antagonist binding or its ability to inhibit a biological response to CCK. This difference in the requirement for sulfation reflects the differences in spatial approximation between the residues of the CCK receptor and position 27 of peptide agonist and antagonist. Indeed, we have shown with photoaffinity-labeling techniques that the  $(pNO_2\text{-Phe})^{27}$  agonist and antagonist label different parts of the CCK receptor. Thus, there are clear structural differences in the conformation of the complexes of CCK receptor with docked agonist and antagonist. Whereas this may reflect distinct differences in docking, contributed to by the structural differences in the antagonist peptide having a dextrorotatory amino acid introduced into its midregion, this probably reflects differences in the conformational changes induced by these structurally related ligands.

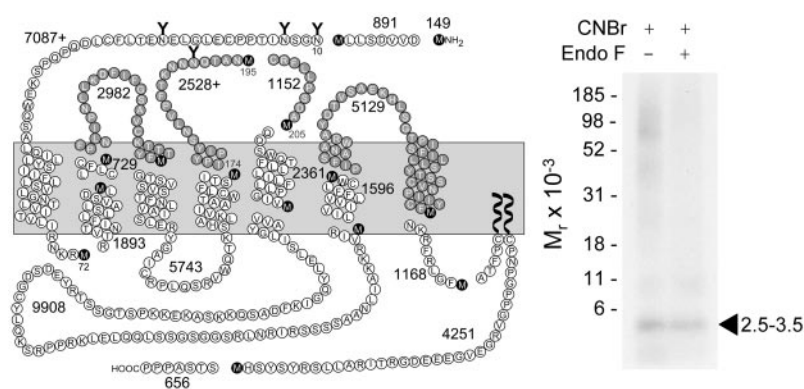
The specificity and high-affinity nature of the interactions between a hormone and its receptor are remarkable and critically important. These allow the hormone, which often circulates in as low as picomolar concentrations, to bind to extremely low receptor densities on target cells, often in the range of a few hundred or thousand molecules per cell. This specificity and affinity come from a constellation of interac-



**Fig. 5.** Photoaffinity labeling of the CCK receptor with the  $(pNO_2\text{-Phe})^{27}$  agonist. The agonist probe specifically labeled the CCK receptor, and the labeling was inhibited by competition with increasing amounts of unlabeled CCK. The labeled receptor migrated at the expected position of  $M_r = 85,000$  to  $95,000$ . When deglycosylated with Endo F, the receptor band shifted to approximately  $M_r = 42,000$ . Absence of labeling of nonreceptor-bearing parental CHO cells is also shown. Right, densitometric analysis of  $(pNO_2\text{-Phe})^{27}$  agonist-labeled CCK receptor with CCK competition with means  $\pm$  S.E.M. from three independent experiments.

**Fig. 6.** Photoaffinity labeling of the CCK receptor with  $(pNO_2\text{-Phe})^{27}$  antagonist. The antagonist probe specifically labeled the CCK receptor, and the labeling was inhibited by competition with increasing amounts of unlabeled CCK. The labeled receptor migrated at the expected position of  $M_r = 85,000$  to  $95,000$ . Right, densitometric analysis of  $(pNO_2\text{-Phe})^{27}$  antagonist-labeled CCK receptor with CCK competition with means  $\pm$  S.E.M. from three independent experiments.





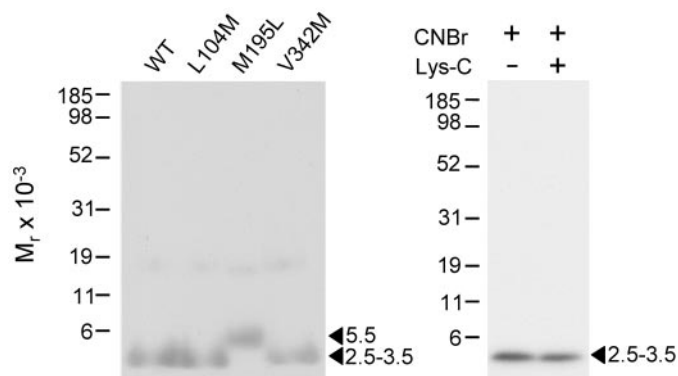
**Fig. 7.** CNBr cleavage of the CCK receptor labeled by the  $(pNO_2\text{-Phe})^{27}$  agonist probe. Left, a diagram of the predicted sites of CNBr cleavage of the CCK receptor, along with sites of N-linked glycosylation. Right, a typical autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr cleavage of the CCK receptor that had been labeled with the  $(pNO_2\text{-Phe})^{27}$  agonist probe. This cleavage yielded a labeled fragment migrating at approximately  $M_r = 2500$  to  $3500$  that did not shift further after deglycosylation with Endo F. This pattern is representative of at least 20 independent experiments. Fragments spanning the three extracellular loops (highlighted in gray) are all possible candidates to represent the domain of labeling with this probe.

tions. With peptide hormones, multiple residues frequently contribute numerous types of interactions, ranging from charge-charge to various types of bonds. Within the constraints provided by the general size, shape, and hydrophobicity of a ligand, its distribution of functionalities contribute to the docking process. Therefore, structurally related ligands, such as those used in this study, would be expected, by first principles, to dock in a similar region of the receptor. Indeed, this is what our previous work demonstrated by photoaffinity-labeling the same region of the CCK receptor using probes analogous to those used in the current study, with each of the agonist, partial agonist, and antagonist probes having a photolabile *p*-benzoylphenylalanine situated at its amino terminus, just outside of the pharmacophoric domain (Dong et al., 1999).

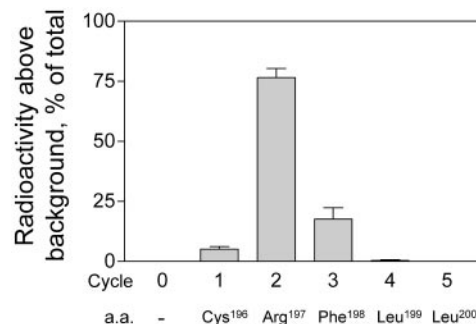
We also know that agonist action of a ligand at a receptor is dependent on a conformational change in the cytosolic face of that receptor, in which the G protein interaction occurs. This conformational change is induced by the binding of the ligand at an ectosurface of the receptor. Consistent with this, one would predict a distinct conformational change induced by the binding of ligands that have distinct biological activ-

ities, even if those ligands are closely related to each other structurally. In the current study, the spatial approximation established to exist between the acidic tyrosine sulfate in the 27 position of CCK and basic Arg<sup>197</sup> within the second extracellular loop region of the CCK receptor is only present in full agonist analogs. The importance of this charge-charge interaction is underscored by the impact sulfation status has on agonist binding affinity and potency. Sulfation is progressively less important as the biological activity of the CCK analog is reduced, to the point that it has no role at all in an antagonist analog. It is particularly interesting that the peptide antagonist that has been extensively used since 1992 when it was introduced by Martinez (Lignon et al., 1987) as a sulfated peptide has the same affinity and biological characteristics as a nonsulfated peptide that can be prepared with much higher yield at a much lower cost.

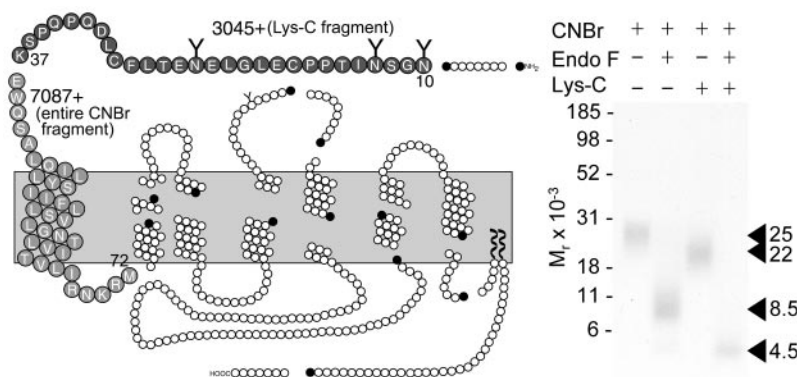
The current work directly confirms the spatial approximation postulated previously to exist between the acidic tyrosine sulfate in the 27 position of CCK and basic Arg<sup>197</sup> within the second extracellular loop region of the CCK receptor (Gigoux et al., 1999; Ding et al., 2002). This interaction was proposed previously from receptor mutagenesis data (Gigoux et al., 1999) and from complementary manipulations of these charged residues in both receptor and ligand (Ding et al., 2002). It is notable that the natural partner for this interaction could not be replaced with a series of distinct isocharged residues and that the complementation could not be successfully mimicked to regain lost function in those studies (Ding et al., 2002). This was supported by extensive



**Fig. 8.** Identification of the domain of labeling by CNBr and endoproteinase Lys-C cleavage of CCK receptor mutants labeled by the  $(pNO_2\text{-Phe})^{27}$  agonist probe. Left, a representative autoradiograph of the CNBr cleavage of the wild-type (WT), L104M, M195L, and V342M CCK receptor mutants expressed on CHO cells. Like cleavage of the labeled wild-type receptor, CNBr cleavage of the labeled L104M and V342M mutants both yielded a labeled band migrating at approximately  $M_r = 2500$  to  $3500$ . However, this band migrated at approximately  $M_r = 5500$  in the M195L receptor mutant, indicating that the fragment Thr<sup>174</sup>-Met<sup>205</sup> (see diagram in Fig. 7) spanning the second extracellular loop contained the domain of labeling with the  $(pNO_2\text{-Phe})^{27}$  agonist probe. Right, the labeled CNBr fragment from the wild-type CCK receptor was not cleaved by endoproteinase Lys-C, indicating that the site of labeling was within the smaller carboxyl-terminal segment Cys<sup>196</sup>-Met<sup>205</sup> (see diagram in Fig. 7) in the second extracellular loop. These data are representative of at least three independent experiments.



**Fig. 9.** Identification of the receptor residue labeled by the  $(pNO_2\text{-Phe})^{27}$  agonist probe. Shown is the radioactive elution profile from cycles of Edman degradation sequencing of the purified CNBr fragment (Cys<sup>196</sup>-Met<sup>205</sup>) resulting from cleavage of the A204C CCK receptor mutant labeled with the  $(pNO_2\text{-Phe})^{27}$  agonist probe. A radioactive peak consistently eluted in cycle 2 that corresponds with covalent labeling of residue Arg<sup>197</sup> of the CCK receptor with this probe. Data represent the means  $\pm$  S.E.M. of three independent experiments.



**Fig. 10.** Localization of the region of the CCK receptor labeled by the  $(pNO_2\text{-Phe})^{27}$  antagonist probe. Left, a diagram of the theoretical sites of cleavage of the CCK receptor, highlighting the CNBr fragment of interest. As shown in the autoradiograph of an SDS-polyacrylamide gel separating the products of digestion (right), CNBr digestion of the CCK receptor labeled with the antagonist probe resulted in a labeled fragment migrating at approximately  $M_r = 25,000$ . Treatment with Endo F after CNBr digestion resulted in a fragment with an apparent  $M_r = 8500$ . This is consistent with labeling of the fragment that includes the first transmembrane segment and part of the amino terminus. Further localization was achieved with endoproteinase Lys-C cleavage. After treatment with this protease and CNBr, the labeled fragment migrated at approximately  $M_r = 22,000$ ; further deglycosylation resulted in a fragment that migrated at an apparent  $M_r = 4500$ . Thus,  $(pNO_2\text{-Phe})^{27}$  antagonist labeled the CCK receptor between Asn<sup>10</sup> and Lys<sup>37</sup>, as highlighted in dark gray in the diagram on the left.

molecular modeling that fully explained these failures, largely on the basis of loss of bonds and rearrangement in conformations. It is encouraging that the current photoaffinity-labeling data support this spatial approximation. Such studies have been criticized previously, with the suggestion that the energy necessary to establish a covalent bond might affect the structures of ligand and receptor and, thereby, disrupt the complex (Escricut et al., 2002). The current work demonstrates that this is not the case, and it validates an extremely useful acidic photolabile residue,  $pNO_2\text{-Phe}$ , for photoaffinity-labeling studies.

This work provides the first insights into the changes in conformation associated with active and inactive states of the CCK receptor. At this stage, there are inadequate experimentally derived constraints in the structure of the amino terminus of this receptor to be able to meaningfully model that region of the receptor. Because it can be truncated without affecting the function of this receptor, it probably acts to cover and protect the peptide-binding domain. As we gain more specific insights such as are provided by the current work, we should be better able to model these distinct states of this receptor and, thereby, better contribute to the rational design and refinement of drugs acting at this important target.

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